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Original article

Didanosine ester prodrugs: Synthesis, albumin binding properties and pharmacokinetic studies in rats

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ABSTRACT

Three half-ester derivatives **10**–**12** of 5′-*O*-2′,3′-dideoxydidanosine (DDI, **1**) have been synthesized. The compounds exhibited excellent correlation between partition coefficients Log *P* and relative *in vitro* bovine serum albumin binding. Using high-performance liquid chromatography–mass spectrometry (LC–MS), DDI (**1**) was quantitatively determined in rat plasma after intravenous injection of the azelaic acid monoester derivative (**11**) of DDI. The pharmacokinetic data obtained for DDI were consistent with literature. The pharmacokinetic profile of **11** showed no significant difference in AUC_{0-360} or curve shape compared to the parent drug DDI (**1**). The data indicate that the prodrug was converted to DDI within minutes after administration. High relative protein binding *in vitro* holds a promise for validity of the concept using more stable linker bonds.

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1. Introduction

Didanosine (5′-*O*-2′,3′-dideoxydidanosine, DDI (**1**)), lamuvidine (**2**) and stavudine (**3**) are nucleoside analog reverse transcriptase inhibitors (NRTIs) [1] for treatment of AIDS [2] and are in routinely clinical use [3] (Fig. 1).

DDI exhibits several pharmacokinetic disadvantages like short plasma half-life (1 h), relative low bioavailability, and has limited penetration into the central nervous system [4,5]. In addition, clinical use of DDI as well as other nucleoside-based inhibitors of viral reverse transcriptase (RT) is associated with severe problems related to side effects and formation of resistant viruses [6,7]. Although several DDI prodrugs have been prepared and evaluated *in vitro* and *in vivo* to overcome some of these problems [8–17] none of these are in routine clinical use as far as we know. The term "drug targeting" encompasses a wide range of different strategies, including fatty acid derivatization. The interactive forces between albumin and fatty acid carboxyl groups are based on ionic forces and hydrophobic interactions between the nonpolar aliphatic chain and cavities between the protein helixes [18–20]. One challenge is that albumin is a catalyst for hydrolysis of various compounds such as esters, amides and phosphate esters. It is reported that the subunit IIA (Sudlow site I) and the subunit IIIA (Sudlow site II) possess significant esterase activity [21,22].

Fatty acid ester derivatives of nucleosidic drugs have been described, e.g. fatty acid ester derivatives of 1-β-D-arabinofuranosylcytosine (ara-C), a deoxycytidine analog with activity against leukemia, were reported to give a significantly lower IC_{50} than the parent drug [23]. However, one disadvantage of the most active compound was low solubility, even in dimethyl sulfoxide (DMSO). Protein binding of these compounds is not known.

Enhancement of protein binding of drugs may have two advantages. In the case of anti cancer drugs, enhanced uptake and accumulation of human serum albumin (HSA) in solid tumors because of enhanced permeability and retention (EPR) of macromolecules have been described [24–26]. Covalent attachment of doxorubicin to albumin resulted in enhanced antitumor efficacy and toxicity; however a potential drawback of this strategy is

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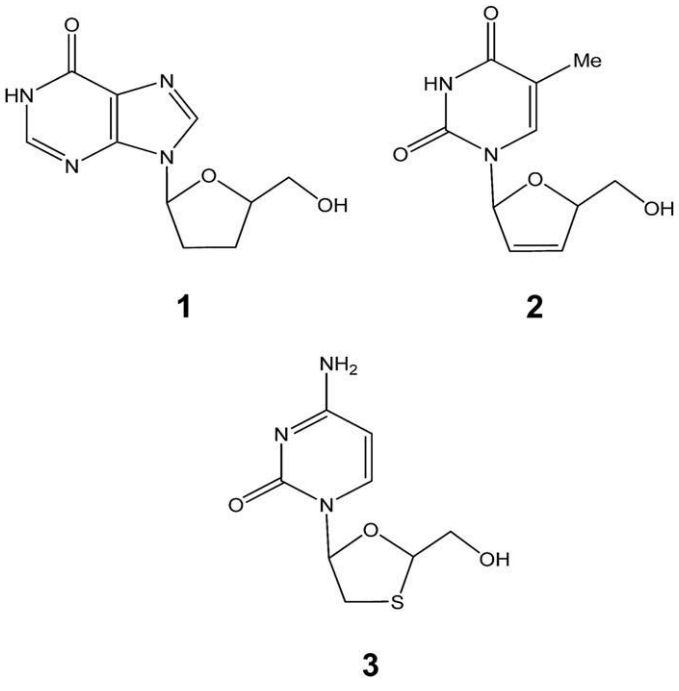


Fig. 1. Molecular structures of didanosine (DDI, **1**), stavudine (**2**) and lamuvidine (**3**).

immunological reactions [27]. The present study describes a new class of diacid half-ester derivatives of drugs [28,29] to mimic fatty acid interaction with serum albumin binding of the drug.

As stated above, DDI exhibits pharmacokinetic parameters which are not optimal [30]. Several methods for the quantitation of DDI from various biological matrixes have previously been reported. Here we report synthesis, characterization and relative protein binding *in vitro* of three diacid derivatives (**9**–**12**) of DDI (**1**), and an evaluation of the systemic exposure of DDI after administration of its monoester derivative of azelaic acid (**11**). This choice of compound for *in vivo* evaluation was based on a) the results of the *in vitro* relative protein binding studies described below and on literature reports providing evidence that the rate of degradation *in vitro* with both rat and pig esterase of long fatty acid chain derivatives of antitumor agent campothecin was less for the ester with chain length C10 than for the ester with chain length C12 [31]. A quantitative method based on a reported study [32] was developed to quantify the DDI level in plasma using LC–MS. Altered pharmacokinetics and distribution of drugs by the means of prodrugs comprising half-esters of long-chain diacids may yield high affinity for albumin, because of the free carboxylic acid functionality in combination with the lipophilic chain. This may mimic albumin binding of free fatty acids. This concept is as far as we can see novel, and might have clinical advantages over the parent drug.

2. Results and discussion

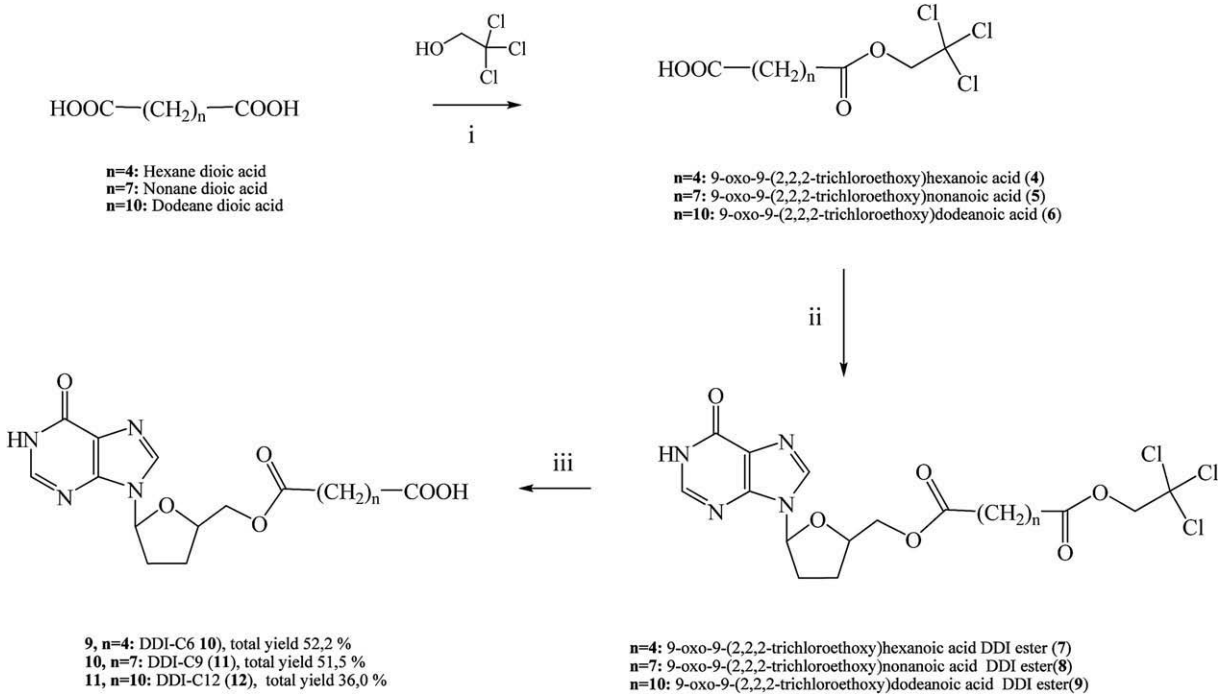
2.1. Chemistry

2.1.1. Synthesis

The syntheses described in this work provided a general, simple method for monoderivatization of long-chain fatty diacids in good yields compared to earlier reports. The method can be used for synthesis of a variety of drug candidates [28,29]. A similar diacid monoderivatization of glucopyranosides has been described earlier, however the method used enzymes and was not general since it was not applicable to shorter diacids [33]. The prodrugs (D–COO–V) consist of an active drug moiety (D), an ester linkage –OOC– and a protein binding moiety (V), see Scheme 1.

2.1.2. In vitro stability test

An indicative stability test of **9**–**12** was performed using a concentration of 0.05 M of both prodrug and *N*-methyl-glucamine



Scheme 1. The synthetic pathways, reaction conditions and chemical structures of DDI prodrugs. i) TsOH, 140 °C Dean–Stark, ii) DDI, DMAP, EDCI, DMF, room temp., ii) Zn, phosphate buffer pH 5.5.

in PBS buffer (Section 3.2.1) at pH 7.4. The samples were stored for 2 weeks at 5, 25 and 40 °C. The initial concentration of each prodrug was compared with the concentration after storage, using HPLC.

No degradation at 5, 25 and 40 °C in the time span of 1 h was observed. After 14 days the degradation was significant. These results indicate that ready to use solutions are not a feasible formulation of these products comprising straight-chain aliphatic ester bonds. A dry powder product (lyophilizate) may be a necessity.

2.1.3. In vitro relative protein binding studies

Estimation of the relative protein binding (PB) of DDI (**1**) and **9**–**12** using literature methods based on ultrafiltration [34,35] was performed in albumin under physiological conditions with respect to pH. The results of the study are summarized in Fig. 2. The relative protein binding correlated well with calculated Log *P* values for **9**–**12**. This also corresponds well with literature data for the free fatty acids [36].

2.2. Pharmacology

Several studies report HPLC–MS–MS analysis of DDI and other nucleoside analogues in plasma and tissue [32,37–40]. In the present study, the concentration of DDI in rat plasma was determined both after injections with DDI and DDI prodrug using a modification of a solid phase extraction (SPE) literature procedure [32] that reported use of stavudine (**2**) as internal standard. However, with our instrumentation, lamuvidine (**3**) was used because of incontrollable MS Na^+ adduct formation with **2**. The analytical method described was applied in the pharmacokinetic study of DDI (**1**) and compound **11** (DDI-C9) through determination of the concentration of DDI in plasma as a function of time. The elimination of DDI from rat plasma was so fast that proper estimates of half-lives were not possible. The systemic exposure of DDI was similar after administration of the prodrug and DDI. AUC_{0-360} was 126 ± 42 and 161 ± 54 mg min/l, respectively ($P = 0.16$). Estimated clearance (CL) was 134 ± 42 and 101 ± 27 ml/min/kg ($P = 0.07$).

2.3. Discussion and conclusions

The similar systemic exposure of DDI after administration of equimolar doses of DDI and the prodrug suggests that the prodrug (**11**) is converted to DDI within minutes in rat plasma. These results are consistent with studies under similar conditions in earlier reports [41] from rat studies using bolus injections of DDI in the same dose regime. It also confirms earlier reports indicating more rapid elimination of DDI in rats than in humans [30,32]. In conclusion, prodrug DDI-C9 did not provide any pharmacokinetic advantages over DDI in rat. The results are supported by literature, indicating that aliphatic ester bonds are rapidly degraded by esterases, possibly reinforced by the esterase activity of HSA. Studies reported by Bundgaard et al. [42,43] concluded that the half-life of prodrugs in an α-chymotrypsin assay showed little difference with different chain lengths in straight-chain esters but increased going from ester > carbonate ester > carbamate ester or sterically hindered esters. Work with more stable biodegradable linker bonds is in progress to evaluate the pharmacokinetic benefits of the concept presented in the present paper, and will be reported later.

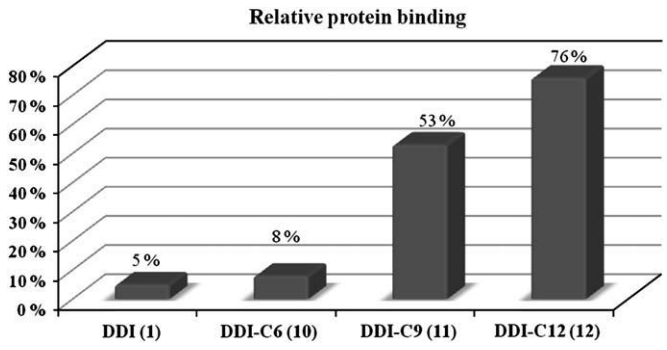


Fig. 2. The relative protein binding of **10**–**12** in HSA solution.

3. Experimental protocols

3.1. Synthesis

3.1.1. Reagents and chemicals

DMSO-d_6 (dimethyl sulfoxide-d_6), chloroform-d_6, 2,2,2-trichloroethanol, azelaic acid, Phosphate Buffered Saline tablets and human serum albumin, essentially fatty acid free, ~99% (agarose gel electrophoresis) as lyophilized powder was purchased from Sigma–Aldrich (St Louis, MO, USA). DMAP (4-Dimethylaminopyridine), formic acid, EDCI (*N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride) and toluene-4-sulfonic were purchased from Fluka. DDI (2′,3′-dideoxyinosine, DDI) was purchased from Dison Chemical Industry Ltd, Shanghai, China. The internal standard stavudine (D4T), HPLC-grade methanol and water and ammonium acetate were purchased from J.T. Baker. Acetonitrile was purchased from Merck. HPLC-grade water was purchased from ElgaPureLab.

3.1.2. General method for the preparation of mono-2,2,2-trichloroetyl-protected DDI half-esters of diacids

To a mixture of diacid **1**–**3** (50 mmol, 5 eq.) and toluene-4-sulphonic acid (10 mmol, 1 eq.) in toluene (100 ml) was added 2,2,2-trichloroethanol (10 mmol, 1 eq.) and stirred at 140 °C with a Dean–Stark trap attached. The reaction mixture was stirred overnight, cooled to 0 °C. Unreacted diacid was filtered off. The filtrate was washed with water (5 × 25 ml), brine (2 × 25 ml), dried over anhydrous $MgSO_4$ and filtered. The organic layer was evaporated *in vacuo* to give the 2,2,2-dioic acids **4**–**6** as yellow oils or white solids. LC–MS Ion trap was used to confirm the identity of the compounds that were used without further purification. A mixture of DDI (**1**, 10 mmol), DMAP (5 mmol) monoprotected diacids **4**–**6** (10 mmol) in DMF (113 ml/g diacid) was stirred at room temperature. *N*-Ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI, 12 mmol) was then added slowly to the mixture. The mixture was stirred overnight at room temperature. The following morning the clear solutions were concentrated *in vacuo*. The products were passed through a silica gel flash column using and MeOH in CH_2Cl_2 20:80 to give the mono trichloroethoxy-mono-DDI diacid derivatives **7**–**9** as yellow oils. LC–MS Ion trap was used to confirm the identity of the compounds that were used in the last step without further purification.

*3.1.3. 6-oxo-6-((5-(6-oxo-1H-Purine-9(6H)-yl)tetrahydrofuran-2-yl)methoxy)hexanoic acid (**10**)*

To a suspension of **7** (4.34 g, 8.76 mmol) in a mixture of 200 ml THF and 40 ml 1 M KH_2PO_4/Na_2HPO_4 buffer at pH 5.5 was added Zn powder (5.6 g) and stirred at room temperature overnight. The mixture was filtered through a pad of kieselguhr and the filtrate was evaporated *in vacuo*. The residue was transferred to a flash column with silica gel and eluted with MeOH in CH_2Cl_2 (5–40%) to give 1.832 g (total yield from **1**: 52.5%) **10** as a white powder, m.p. 67–70 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.49 (s, 1H, H-1), 8.26

(s, 1H, H-2), 8.08 (s, 1H, H-8), 6.24 (t, 1H, $J = 4.05$ Hz, H-1′), 4.38–4.27 (m, 1H, H-4′), 4.23 (dd, 1H, $J = 11.9$, 3.3 Hz, H-5$_b$′), 4.16 (dd, 1H, $J = 11.7$, 5.8 Hz, H-5$_a$), 2.50–2.00 (m, 8H, 4H 2′,3′ and 4H α carbonyl), 1.6–1.4 (m, 4H, fatty acid chain). ^{13}C NMR (75 MHz, DMSO-d_6): δ 176.8, 172.6, 156.6, 147.7, 138.2, 124.3, 84.3, 78.5, 64.9, 34.1, 33.1, 26.0, 24.4, 24.1. MS *m/z*: (ES+, Ion Trap): 137.1 [Purine + H]$^+$, 365.3 [M + H]$^+$, 387.2 [M + Na]$^+$, 403.1 [M + K]$^+$, 423.1 [M + AcO]$^+$. MS *m/z*: (TOF MS ES+) 387.2 [M + Na]$^+$. HRMS (TOF MS ES+) for $C_{16}H_{20}N_4O_6$ [M + Na]$^+$: calcd, 387.1298; found, 387.1280.

3.1.4. 9-oxo-9-((5-(6-oxo-1H-Purine-9(6H)-yl)tetrahydrofuran-2-yl)methoxy)nonanoic acid (**11**)

To a suspension of **8** (3.64 g, 6.7 mmol) in a mixture of 150 ml THF and 30 ml 1 M KH_2PO_4/Na_2HPO_4 buffer at pH 5.5 was added Zn powder (2.0 g) and stirred at room temperature overnight. The mixture was filtered through a pad of kieselguhr and the filtrate was evaporated *in vacuo*. The residue was transferred to a flash column with silica and eluted with MeOH (30%) in CH_2Cl_2 to give 1.843 g (total yield from **2**: 51.5%) **11** as a white powder, m.p. 76–79 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.45 (s, 1H, H-1), 8.24 (s, 1H, H-2), 8.08 (s, 1H, H-8), 6.24 (t, 1H, $J = 4.2$ Hz, H-1′), 4.37–4.27 (m, 1H, H-4′), 4.23 (dd, 1H, $J = 11.9$, 3.3 Hz, H-5$_b$′), 4.15 (dd, 1H, $J = 11.7$, 5.8 Hz, H-5$_a$) 2.50–2.52 (m, 8H, 4H 2′,3′ and 4H α carbonyl), 1.60–1.10 (m, 10H, fatty acid chain). ^{13}C NMR (75 MHz, DMSO-d_6): δ 177.3, 172.7, 156.6, 147.7, 145.7, 138.1, 124.3, 84.3, 78.5, 64.8, 35.4, 33.2, 31.2, 28.6, 28.4, 28.3, 26.0, 25., 24.3. MS *m/z*: (ES+, Ion Trap): 137.1 [Purine + H]$^+$, 407.3 [M + H]$^+$, 429.2 [M + Na]$^+$, 445. [M + K]$^+$, 465.1 [M + AcO]$^+$. MS *m/z*: (TOF MS ES+) 407.2 [M + H]$^+$, 429.2 [M + Na]$^+$. HRMS (TOF MS ES+) for $C_{19}H_{26}N_4O_6$ [M + Na]$^+$: calcd, 429.1798; found, 429.1744.

3.1.5. 2-oxo-12-((5-(6-oxo-1H-purine-9(6H)-yl)tetrahydrofuran-2-yl)methoxy)dodecanoic acid (**12**)

To a suspension of **9** (3.084 g, 5.32 mmol) in a mixture of 125 ml THF and 25 ml 1 M KH_2PO_4/Na_2HPO_4 buffer at pH 5.5 was added Zn powder (3.3 g) and stirred at room temperature overnight. The mixture was filtered through a pad of kieselguhr and the filtrate was evaporated *in vacuo*. The residue was transferred to a flash column with silica and eluted with MeOH (5–40%) in CH_2Cl_2 as eluent system to give 1.47 g (total yield from **3**: 36.0%) **12** as a white powder, dec. 112–120 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.26 (s, 1H, H-2), 8.10 (s, 1H, H-8), 6.31–6.22 (t, 1H, $J = 3.9$ Hz, H-1′), 4.40–4.28 (m, 1H, H-4′), 4.25 (dd, 1H, $J = 11.9$, 2.9 Hz, H-5$_b$′), 4.18 (dd, 1H, $J = 11.8$, 5.8 Hz, H-5$_a$′), 2.26–2.04 (m, 8H, 4H 2′,3′ and 4H α carbonyl), 1.58–1.07 (m, 16H, fatty acid chain). ^{13}C NMR (75 MHz, DMSO-d_6): δ 172.7, 156.6, 147.7, 145.7, 138.1, 124.4, 84.4, 78.5, 64.9, 48.5, 35.4, 33.3, 31.3, 28.9, 28.9, 28.9, 28.7, 28.4, 26.0, 25.1, 24.4. MS *m/z*: (ES+, Ion Trap): 137.1 [Purine + H]$^+$, 449.2 [M + H]$^+$, 471.1 [M + Na]$^+$, 487.0 [M + K]$^+$, 507.1 [M + AcO]$^+$. MS *m/z*: (TOF MS ES+): 449.3 [M + H]$^+$. HRMS (TOF MS ES+) for $C_{22}H_{32}N_4O_6$ [M + Na]$^+$: calcd, 471.2198; found, 471.2211.

3.2. In vitro studies

3.2.1. Preparation of solutions

Phosphate Buffered Saline, pH 7.4 (PBS). Fresh 200 ml PBS solution, pH 7.4, was prepared by dissolving one Phosphate Buffered Saline tablet in purified water (200 ml). The buffer contained 0.01 M phosphate buffer (0.0081 M Na_2PO_4 + 0.0015 M KH_2PO_4), KCl (0.0027 M) and NaCl (0.137 M). *Human Serum Albumin 4% (HSA) in Phosphate Buffered Saline (PBS)*: HSA (400 mg) was dissolved in PBS and diluted to 10 ml with PBS. *DMSO stock solutions of DDI and DDI derivatives in DMSO:* 5 mg of DDI (**1**) and compounds **10**–**12** were each dissolved in DMSO (1 ml) in 0.5% (w/v) (5 mg/ml) concentrations. *Injection solutions*: DDI (**1**, 118.1 mg, 0.5 mmol) and DDI-C9 (203.2 mg, 0.5 mmol) were each dissolved in vials and diluted with PBS buffer (to 10 ml), which gave the desired concentration of 0.05 M solution. The solutions were filtered through a sterile 0.20 μm pore size membrane filter (Advantech Dismic-25).

3.2.2. Utrafiltration

Ultrafiltration was performed on a Heraeus Sepatech Minifuge using a Tibrofix VFI Electronic Whirl mixer and Eppendorf Thermomixer Comfort Incubator with shaker. The filter device was a Centricon Ultracel YM-10 Regenerated cellulose 10.000 MWCO, volume 1.0 ml, rotation speed 4000 rpm, ultrafiltration time 60 min and temperature 37 °C.

3.2.3. Indicative stability

A simplified indicative stability program for **9**–**12** was performed using a concentration of 0.05 M of both the prodrug and *N*-methyl-glucamine in PBS buffer (Section 3.2.1) at pH 7.4. The samples were stored for 2 weeks at 5, 25 and 40 °C. The initial concentration of each prodrug was compared with the concentration after storage, using HPLC.

3.2.4. In vitro relative protein binding (% PB)

The method used herein to determine % PB is a slight modification of published methods [34,35]. One vial containing 1.0 ml PBS and one vial containing 1.0 ml HSA buffer were each added an equal amount of DMSO stock solution of substances **10**–**12**, giving concentrations of approximately 0.050 μmol/ml. The vials were capped and mixed for about 30 s on a whirl mixer. About 0.2 ml of the content in the PBS vial was transferred to another vial to serve as a control for absorbance of the compound to the membrane. The vials were then placed for incubation on an Eppendorf Thermomixer Comfort keeping 37 °C for 1 h. After incubation the vials were immediately transferred to a Centricon filter device and centrifuged at 4000 rpm swing out rotor keeping 37 °C for 1 h. HPLC analyses were repeated twice for each vial. The relative protein binding (% PB) for the different derivatives was calculated using the following equation:

$$\frac{\dfrac{\mathrm{AUC_{PBS}}}{\mathrm{Substance_{PBS}}} - \dfrac{\mathrm{AUC_{HSA}}}{\mathrm{Substance_{HSA}}}}{\dfrac{\mathrm{AUC_{PBS}}}{\mathrm{Substance_{PBS}}}} \times 100 = \%\mathrm{PB}$$

The absolute HPLC peak area of the substance in both the filtrate and retentate was used to calculate the concentrations of substance after centrifugation. For each of the products, AUC_{PBS} is the measured peak area (absolute values) of the substance in the filtrate of the centrifuged buffer solution, AUC_{HSA} is the peak area of the substance in the filtrate of the centrifuged human serum albumin solution, $Substance_{PBS}$ is the amount of substance applied to the buffer solution and $Substance_{HSA}$ is the amount of substance applied to the human serum albumin solution. During centrifugation, about 0.5 ml of filtrate was obtained. To eliminate volume effects on the binding equilibria between experiments, the experimental conditions for each experiment were carefully kept constant, reducing relative variation. Absorbance of the compound to the membrane was checked using the 0.2 ml aliquot from the PBS vial as mentioned above.

3.3. Instruments

3.3.1. NMR

^{1}H and ^{13}C NMR spectra were recorded at 300 MHz and 75 MHz respectively on a Bruker Avance DPX300 instrument with BACS

automatic sample changer. All experiments were performed at 25 °C. DMSO-d_6, $CDCl_3$-d and TMS were used as references. Chemical shifts for the solvents used were reference peaks: DMSO-d_6: ^{1}H δ 2.50 (q), ^{13}C δ 39.43 (sep), $CDCl_3$-d: ^{1}H δ 7.26 (s) and ^{13}C δ 77.0 (t).

3.3.2. LC–MS

Two different LC–MS systems were used in the different analytical experiments. For structural confirmation and monitoring of starting materials and synthesized compounds, an Agilent 6300 Series Ion Trap equipped with a 1200 Series LC system and ChemStation LC 3D systems software, running in positive ion mode (ES+) in MRM mode were used. Solutions (10 μg/ml) of stavudine (**2**), lamuvidine (**3**) and DDI (**1**) dissolved in ammonium acetate buffer were injected directly into the capillary of the ESI source at a constant flow rate of 0.8 ml/h using a KD scientific syringe pump. The ESI capillary potential was set to 4.5 kV, the nebulizer pressure was set to 15 psi, the drying gas flow rate was set to 4 L/min and the drying gas temperature was set to 250 °C. Helium was used as collision gas. Because of problems with Na^+ adduct formation of the internal standard **3** with the ion trap, the system used for the bioanalytical studies of rat plasma was an Agilent 6410 triple quadrupole MS equipped with a 1200 Series LC system and Mass Hunter Workstation software for qualitative and quantitative analyses. The chromatographic conditions in the bioanalytical method were optimized to ensure protonation of DDI (**1**) and the chosen internal standard **3**. The mobile phases consisted of 10 mM aqueous ammonium acetate buffer pH 6.3 and methanol at 0.5 ml/min. The phase front was observed at 1.2 min and this was used to determine the retention factors k' for (**3**) and DDI (**1**) using the different gradients. The runtime was 10 min for each gradient. The gradient of choice gave a $k_{DDI} = 5$ and a $k_{\mathbf{3}} = 3.7$ and $\alpha = k_{DDI}/k_{\mathbf{3}} = 1.4$. Total ion scan mass spectra of DDI (**1**) and DDI-C9 (**11**) were obtained operating the triple quad in positive mode. DDI-C9(**11**) showed the protonated molecule $[M+H]^+$ (m/z 407), **3** showed the protonated molecule, $[M+H]^+$ (m/z 230) and DDI-C9 (**11**) showed $[M+H]^+$ (m/z 407). Product ion scan mass spectra were obtained for the three compounds. The most abundant molecule fragment was the protonated hypoxanthine, m/z 137 for DDI (**1**) and DDI-C9 (**11**). The most abundant fragment was the protonated 4-amino-pyrimidinone m/z 112 for **3**. These ions were the result of fragmentation at the glycosidic bond in **1**, **11** and **3**. For required selectivity in the analytical method, the mass spectrometer was then set up in multiple reaction-monitoring (MRM) mode, to monitor the following transitions: m/z 237 (Q1) → m/z 137 (Q3), m/z 407 (Q1) → 137 (Q3) and m/z 230 (Q1) → m/z 112 (Q3) for DDI (**1**), DDI-C9 (**11**) and (**3**) respectively.

The most diluted samples for the calibration curve standards were 3.2 ng/ml. The average signal to noise ratio for the three parallels was 3363. The FDA guidelines for bioanalytical method validation state that the analyte response at the LLOQ should be at least 5 times the response compared to blank response. The signal to noise ratio suggests that the method is able to go way further down in concentration, may be picogram levels. The calibration curve showed linear response ($R^2 = 97.7\%$) over the range of prepared standard samples 40 ng/ml–10 μg/ml.

3.4. Bioanalytical methods

3.4.1. Administration of drug and sample collection

All procedures involving animals were performed according to protocols approved by the National Animal Research Authority and conducted according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Male and Female Rowet nu/nu rats bred at the nude rodent facility at the Norwegian Radium Hospital were used. The animals were maintained under specific pathogen-free conditions, with filtered and humidified air. Test compounds were administered by intravenous bolus tail vein injection in 18 rats with average weight 225 g. Two groups of 9 rats received a dose of 0.065 mol/kg of DDI (**1**) or DDI-C9 (**11**, 15 mg/kg) respectively. At 5 min, 15 min, 30 min, 1 h, 3 h and 6 h, blood samples were taken by heart puncture from groups of three animals, anesthetized with 4% sevoflurane (Sevoflurane, Baxter, Deerfield, IL) in a mixture of N_2O and O_2. Immediately thereafter the animals were sacrificed by an i.p. injection of 100 mg/kg pentobarbital. Plasma samples were prepared by immediate centrifugation at 1000 *g* for 5 min and stored at −80 °C. Area under the plasma concentration versus time curve (AUC) was calculated by the trapezoidal method. Concentration at time zero was set to be the same as at 5 min and absolute concentrations were used even below LLOQ. Clearance (CL) was calculated as according to the formula, CL = Dose/AUC.

3.4.2. Preparation of standard solutions

Stock solutions of DDI (**1**) were prepared by dissolving DDI (50 mg) in optima water (10 ml). The internal standard (IS) stock solution was prepared by dissolving **3** (50 mg) in optima water (10 ml). The IS solution was prepared by serial dilution of stock solution giving 25 μg/ml.

3.4.3. Calibration curves and quantification

Standards for calibration curves were prepared by spiking of drug free rat plasma (990 μl) with DDI (10 μl 5 mg/ml stock solution). The spiked plasma was then serially diluted with blank plasma giving the concentrations 10, 2, 0.4, 0.08, 0.016, 0.0032 μg/ml. Zero- and blank samples were also prepared. The samples were vortex mixed and prepared as described below.

3.4.4. Sample preparation

Rat plasma (100 μl) and lamuvidine (**3**) internal standard (10 μl, 25 μg/ml) were loaded directly on a Sep-Pak Plus C18 cartridge (Waters) preconditioned with 1 ml methanol, followed by 10 mM ammonium acetate buffer (1 ml, pH 6.3). The samples were then washed with ammonium acetate buffer/methanol (95:5), eluted with 1 ml methanol directly into an HPLC vial. The methanol was evaporated using a Savant SVC 200 speed vac with an RT400 Refrigerated Condensation Trap. The samples were reconstituted in optima water (50 μl), 20 μl was injected in the HPLC–MS–MS system.

3.4.5. Bioanalytical LC method

The mobile phase used was a 10 mM ammonium acetate (pH adjusted to 6.3 with acetic acid) and methanol gradient (Table 1) with flow rate 0.50 ml/min at ambient temperature. The chromatographic separation was achieved on a Zorbax SB-Aq (3.5 μm, 3.0 mm × 100 mm) analytical column using an injection volume of 20 μl.

Table 1
Eluents for the LC–MS analyses of products **1**–**12**. Eluent A: 10 mM ammonium acetate buffer pH 6.3, eluent B: methanol.

Time (min)	Mobile phase (% v/v)	
	A	B
0 → 10	95 → 50	5 → 50
10 → 12	50 → 30	50 → 70
12 → 13	30 → 95	70 → 5
13 → 18	95	5

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